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Agar extraction process for *Gracilaria cliftonii* (Withell, Millar, & Kraft, 1994)

Vivek Kumar *, Ravi Fotedar

Curtin Aquatic Research Laboratories, Faculty of Science and Engineering, Curtin University of Technology, GPO Box U1987, Perth, WA, Australia

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ABSTRACT

Agar extraction process was developed for *Gracilaria cliftonii* by investigating the effects of various extraction variables and alkali treatments on agar yield and properties. The tested variables were soaking time, soaking temperature, seaweed to water ratio of, extraction temperatures and extraction time. Alkali treatments were carried out in alkali concentrations of 1%, 2%, 3% and 5% in a water bath at 60, 70 and 80 °C prior to agar extraction. The results showed that agar yield was significantly affected by all the tested variables. The agar yield was maximised when extraction process was carried out with 1 h soaking time at 30 °C with seaweed to water ratio of 1:150 and extracted for 3 h at 100 °C. The alkali-temperature combinations significantly influenced agar yield and properties. Irrespective of temperature, alkali treatments at 3% and 5% significantly increased the gel strength.

1. Introduction

Agar, a hydrophilic colloid is the first phycocolloid used in the human food industry (Armisen & Galatas, 2000) accounting for 80% of its consumption, while the remaining 20% is used for biotechnological applications. Agars with gel strengths greater than 700 g/cm^2 in a 1.5% solution are referred as high quality agars and are in demand in the international market (Armisen, 1995). The application of the agar in different products depends on its chemical composition (sulphate, methoxyl, sugar contents) which can be significantly affected by the variables used in the extraction process (Pereira-Pacheco, Robledo, Rodriguez-Carvajal, & Freile-Pelegrin, 2007).

Gracilaria species are the major agar source worldwide (McHugh, 2003) and numerous studies have been conducted on their agar yield and quality from different geographical areas (Doty, Santos, & Sin, 1983; Falshaw, Furneaux, Pickering, & Stevenson, 1999; Freile-Pelegrin & Murano, 2005; Marinho-Soriano, 2001; Marinho-Soriano, Bourret, de Casabianca, & Maury, 1999; Santos & Doty, 1983). Seven species of *Gracilaria* have been identified in Australia, five of these are found in Western Australia: *G. cliftonii*, *G. blodgetti*, *G. perissana*, *G. canaliculata and G. flagelliformis* (Huisman, 2000). *G. cliftonii* is distributed from Geraldton to Esperance and is high in agar content (52%) (Byrne, Zuccarello, West, Liao, & Kraft, 2002).

The general methodology established for agar extraction consists of leaching the dry *Gracilaria* in boiling water, filtering off the extract, and separating the agar by freezing and thawing to eliminate the water (Armisen & Galatas, 1987). Although the general steps in the agar extraction process from *Gracilaria* species are known (Andriamananatonio, Chambat, & Rinaudo, 2007; Arvizu-Higuera, Rodríguez-Montesinos, Murillo-Álvarez, Muñoz-Ochoa, & Hernández-Carmona, 2008; Freile-Pelegrin & Murano, 2005; Li et al., 2009; Marinho-Soriano, 2001; Orduña-Rojas et al., 2008; Pereira-Pacheco et al., 2007; Tako, Higa, Medoruma, & Nakasone, 1999), the extraction variables and methodologies differ. Therefore, it is necessary to standardise the extraction process to optimise the agar yield and quality. A notable reduction on agar yield and decrease in gel strength are frequently associated with elevated extraction temperatures and prolonged extraction time (Arvizu-Higuera et al., 2008; Hurtado-Ponce, 1992; Pereira-Pacheco et al., 2007). The influence of extraction temperature and extraction time on agar characteristics has been reported for *Gracilaria* species (Arvizu-Higuera et al., 2008; Hurtado-Ponce, 1992; Pereira-Pacheco et al., 2007) but no report is available on *G. cliftonii*.

Generally, *Gracilaria* species produces agars with low quality due to their high sulphate concentrations. However, the gel properties of many *Gracilaria* agars can be improved by alkali treatment, which converts L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Duckworth, Hong, & Yaphe, 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas et al., 2008), which is responsible for the enhancement of the gel forming ability. Alkali treatment variables like alkali concentration, heating time, temperature are reported to affect the yield and quality of agar from other *Gracilaria* species (Andriamananatonio et al., 2007; Armisen & Galatas, 1987; Arvizu-Higuera et al., 2008; Li et al., 2009; Orduña-Rojas et al., 2008). Alkali treatment

* Corresponding author. Tel.: +61 401 253 223; fax: +61 8 9266 4422.
E-mail address: vivek_kumar_in@yahoo.com (V. Kumar).

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of *Gracilaria* species must be adapted for each species and variables like temperature and alkali concentration must be adjusted to obtain as much desulphation as possible, while still avoiding the yield losses caused in the treatment (Armisen & Galatas, 1987; Orduña-Rojas et al., 2008).

G. cliftonii is an economically important resource due to its high agar yield of 52%db (Byrne et al., 2002) but agar characteristics of this species are yet to be reported. In addition, no studies have been reported on the alkali treatment of *G. cliftonii*. The agar extraction process of *G. cliftonii* was evaluated to maximise the agar yield by investigating the effects of five variables *viz.* soaking time, soaking temperature, seaweed–water ratio and time and temperature of extraction. The aim of the current research was to investigate the effects of extraction variables on chemical and physical properties (gel strength, gelling temperature, melting point and sulphate content) of agar. In addition, the effect of alkali treatments on the agar characteristics of *G. cliftonii* at different soaking temperatures was also investigated. This information provides the basis to standardise the procedure of agar extraction from *G. cliftonii* to obtain agar with characteristics accepted by industry.

2. Experimental

2.1. Sample preparation

G. cliftonii fronds were collected from shallow creeks around Point Peron, Shoalwater Islands Marine Park (32° 17′ S, 115° 42′ E), Perth, WA by free diving and transported in containers filled with ocean water to Curtin Aquatic Research Laboratory (CARL). *G. cliftonii* samples were washed with tap water to remove impurities. When the seaweed was free from visible impurities it was then considered as 'clean seaweed'. The 'clean seaweed' sample was oven dried for 8 h at 60 °C and stored in sealed plastic bags for agar extraction.

2.2. Agar extraction process

2.2.1. Soaking time

First lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) each and nine samples from these 12 samples were soaked for 1, 2 and 3 h at room temperature (25 °C) in triplicate to hydrate. The remaining three samples were used as control and were not soaked (0 h). Extraction was carried out by boiling all samples for 2.5 h in 250 mL conical flasks with distilled water at 90 °C in a water bath.

2.2.2. Soaking temperature

Second lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) each and nine samples from these 12 samples were soaked for 2 h at three different temperatures (30, 35 and 40 °C) in triplicate. Three remaining samples were soaked at room temperature (25 °C) and used as control in triplicate. Extraction was carried out by boiling all samples for 2.5 h in 250 mL conical flasks with distilled water at 90 °C in a water bath.

2.2.3. Seaweed to water ratio

Third lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) each and soaked for 2 h at 30 °C in different volume of water to represent variable seaweed–water ratios. These four different seaweed–water ratios were 1:50, 1:100, 1:150 and 1:200. All of them were used in triplicate and then transferred to water bath for agar extraction. Extraction was carried out by boiling all the samples for 2.5 h in 250 mL conical flasks with distilled water at 90 °C in a water bath.

2.2.4. Extraction temperature

Fourth lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) each and soaked for 2 h at 30 °C with seaweed–water ratio of 1:150. Extraction was carried out at four different temperatures of 70, 80, 90 and 100 °C in triplicate by boiling the samples for 2.5 h in four water baths.

2.2.5. Extraction time

The fifth and last lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) each and soaked for 2 h at 30 °C with seaweed–water ratio of 1:150. Extraction from the samples was carried out at 100 °C at five different times of 1.0, 1.5, 2.0, 2.5, 3.0 h in triplicate in a water bath.

2.2.6. Agar yield

The extracts from all the lots were filtered using a three-ply cheesecloth and were transferred to plastic containers (500 mL). The filtrate was frozen overnight, thawed and oven dried at 60 °C for 24 h and weighed. Quantity of agar was determined in terms of agar yield expressed as percentage dry basis and calculated from the following equation

$$\text{Agar Yield} = [(\text{Dry weight of agar(g)}/\text{Dry weight of seaweed(g)})] \times 100 \quad (1)$$

2.2.7. Alkali preparation and treatments

Four concentrations of 1%, 2%, 3% and 5% alkali were prepared dissolving analytical grade sodium hydroxide (NaOH) (Sigma®) in distilled water. The dried samples of *G. cliftonii* (5 g db) in triplicate were soaked in each alkali concentrations of 1%, 2%, 3% and 5% for 3 h at room temperature in 250 mL conical flask. The flasks were then placed for 1 h in a water bath at 60, 70 and 80 °C, respectively. After alkali treatment, the samples were washed with running tap water for 1 h to remove excess alkali. Extraction was carried out by boiling the sample for 2.5 h in 250 mL of distilled water at 7.0–7.5 pH. The extracts were filtered using three-ply cheesecloth and transferred to plastic containers (500 mL). The filtrate was allowed to gel at room temperature, frozen overnight and thawed. Finally, the agar was oven dried for 24 h at 60 °C, cooled and weighed to calculate percent agar yield on dry weight basis (%db) and calculated from Eq. (1).

2.3. Agar properties

The dried agar from extraction process variables and alkali treatments was reconstituted in 250 mL glass beakers with distilled water to obtain a 1.5% w/v agar solution. The hot gel was poured into 50 mL sterile plastic containers and remaining in a glass test tube (2.3 cm diameter, 16.5 cm height) to determine gel strength, melting point and gelling temperature, of the agar. The physical and chemical properties (gel strength, melting temperature, gelling temperature and sulphate content) of the agar were determined after curing gel for 24 h at 25 °C.

Gel strength was determined as described by Marinho-Soriano and Bourret (2005) with minor modifications. A texturometer *TX-2i* with a 1-cm^2 plunger at a speed of 1 mm/s to depth 5 mm was used on gel in 50 mL sterile containers. Melting point and gelling temperature were determined using techniques described by Freile-Pelegrin and Murano (2005) with minor modifications. Melting point of the gel in test tubes was measured by placing a glass bead (5 mm diameter) on the gel surface. The test tube rack with test tube was transferred to the water bath at boiling temperature. The melting point was recorded with a digital thermometer when the bead sank into the solution. Same test tubes were kept at room temperature to measure the gelling temperature. The tubes were tilted up and down in a water bath at room temperature until the glass bead ceased moving. The gel temperature in the tube

was immediately measured by introducing a digital thermometer into the agar gel.

Sulphate was determined using modified AOAC Gravimetric method (AOAC, 1995) with minor modifications. Dry agar sample of 0.5 g was transferred into standard digestion tubes with 10 mL of concentrated HNO_3. The digestion tubes were transferred into digester set at 123 °C for 30 min to have the final volume of digest as 2–3 mL. After cooling the samples in fume hood, 2–3 drops of 40% HCHO solution were added to reduce the excess HNO_3. The mixture was filtered into 250 mL conical flask and 0.5 mL concentrated HCl was added followed by distilled water to bring the volume to 200 mL. The solution was heat to boiling and 10 mL of 0.25 M $BaCl_2$ was added dropwise with constant stirring for 5 min and kept aside for 5 h in warm place. The $BaSO_4$ precipitate solution was filtered with filter paper (Whatman No. 5) and precipitate was ashed in crucibles in muffle furnace at 700 °C for 1 h. The crucibles were transferred to desiccator for cooling and weighed to determine the weight of $BaSO_4$. The percentage sulphate was calculated from the following equation

$$\text{Sulphate}(\%\text{db}) = [(41.16 \times \text{weight of } BaSO_4)/\text{sample weight}] \times 100 \quad (2)$$

2.4. Statistical analysis

Statistical software, SPSS 16.0 for Windows was used to analyse all data. The data were tested for homogeneity of group variance (Levene's). Agar yield and properties were subjected to one-way ANOVA and least significant difference (LSD) post hoc test for significant differences at *p*-value of less than 0.01 and 0.05. The correlation between agar properties were subjected to independent sample *t*-test at significance level of $p < 0.05$. Microsoft Excel 2007 was used to determine the correlations and regression equation between two parameters and with respect to third parameter.

3. Results

3.1. Agar extraction variables

Soaking time and temperature significantly influenced agar yield and gelling temperature (Tables 1 and 2). The maximum agar yield was obtained with soaking time of 1 and 2 h and was significantly higher ($p < 0.05$) than 3 h soaking time. Gelling temperature of agar with 3 h soaking time was significantly higher ($p < 0.05$) than other soaking times. Agar yield showed strong negative correlation ($r^2 = -0.8$, $p < 0.05$) with sulphate content with increasing soaking time. Agar yield at soaking temperature of 30 °C was significantly higher ($p < 0.05$) than other soaking temperatures. Gelling temperature of agar at 30 °C was significantly higher ($p < 0.05$) than 40 °C soaking temperature. The sulphate content of agar at 35 °C was significantly higher ($p < 0.05$) than control.

Table 1
Agar yield and properties (mean ± standard error) from *Gracilaria cliftonii* at soaking time 0, 1, 2 and 3 h.

	Soaking time (h)			
	0	1	2	3
Agar yield (%db)	59.8 ± 1.0^{a,b}	61.2 ± 0.1^{a}	61.2 ± 1.0^{a}	54.9 ± 2.6^{b}
Gel strength (g/cm^2)	171.8 ± 8^{a}	159.4 ± 14^{a}	170.9 ± 8^{a}	180.8 ± 12^{a}
Melting point (°C)	86.5 ± 0.9^{a}	87.1 ± 1.5^{a}	86.7 ± 0.7^{a}	85.8 ± 0.6^{a}
Gelling temperature (°C)	33.1 ± 0.2^{a}	33.1 ± 0.3^{a}	33.2 ± 0.3^{a}	37.9 ± 0.5^{b}
Sulphate (%db)	7.1 ± 0.3^{a}	6.9 ± 0.5^{a}	6.8 ± 0.0^{a}	7.1 ± 0.9^{a}

Different letters (a, b) indicates significant differences at a level of $p < 0.05$.

Table 2
Agar yield and properties (mean ± standard error) from *Gracilaria cliftonii* at soaking temperature of 25, 30, 35 and 40 °C.

	Soaking temperature (°C)			
	25	30	35	40
Agar yield (%db)	52.7 ± 0.2^{a}	62.3 ± 0.7^{b}	49.7 ± 1.9^{a}	51.3 ± 1.2^{a}
Gel strength (g/cm^2)	126.3 ± 6^{a}	119.0 ± 4^{a}	117.7 ± 1^{a}	123.3 ± 5^{a}
Melting point (°C)	81.8 ± 3.5^{a}	86.7 ± 0.3^{a}	88.0 ± 2.0^{a}	86.5 ± 1.7^{a}
Gelling temperature (°C)	35.0 ± 1.2^{a,b}	35.5 ± 1.0^{a}	34.9 ± 1.3^{a,b}	32.0 ± 0.4^{b}
Sulphate (%db)	5.1 ± 1.1^{a}	7.5 ± 0.7^{a,b}	8.2 ± 0.9^{b}	7.7 ± 0.5^{a,b}

Different letters (a, b) indicates significant differences at a level of $p < 0.05$.

Table 3
Agar yield and properties (mean ± standard error) from *Gracilaria cliftonii* with seaweed–water ratio of 1:50, 1:100, 1:150 and 1:200.

	Seaweed to water ratio			
	1:50	1:100	1:150	1:200
Agar yield (%db)	52.6 ± 0.4^{a}	50.5 ± 0.7^{b}	57.8 ± 0.2^{c}	53.7 ± 0.2^{a}
Gel strength (g/cm^2)	133.0 ± 9^{a,b}	124.3 ± 5^{a}	143.1 ± 11^{a,b}	159.9 ± 14^{b}
Melting point (°C)	85.7 ± 1.2^{a}	87.1 ± 0.8^{a}	85.6 ± 0.4^{a}	84.9 ± 1.8^{a}
Gelling temperature (°C)	34.7 ± 0.7^{a}	33.2 ± 0.2^{a}	33.1 ± 0.4^{a}	34.8 ± 1.5^{a}
Sulphate (%db)	5.9 ± 1.5^{a}	7.2 ± 0.7^{a}	7.1 ± 1.3^{a}	4.8 ± 0.9^{a}

Different letters (a, b, c) indicates significant differences at a level of $p < 0.05$.

Gel strength showed negative correlation ($r^2 = -0.7$, respectively, $p < 0.05$) with sulphate content with increasing soaking temperature.

Agar yield and gel strength were significantly influenced with various seaweed–water ratios (Table 3). The agar yield with seaweed–water ratio of 1:150 was significantly higher ($p < 0.05$) than other ratios, while seaweed–water ratio of 1:100 was significantly lower ($p < 0.05$). Gel strength of agar with seaweed–water ratio of 1:100 was significantly lower ($p < 0.05$) than seaweed–water ratio of 1:200. Gel strength was negatively correlated ($r^2 = -0.8$, $p < 0.05$) with melting point while gelling temperature was negatively correlated ($r^2 = -0.9$, $p < 0.05$) with sulphate content with increasing seaweed–water ratio.

The effect of extraction temperature and time on agar yield and properties is shown in Tables 4 and 5. Maximum agar yield was obtained at extraction temperature of 100 °C and minimum at 70 °C. Agar yield and melting point showed strong positive correlation ($r^2 = 0.98$ and $r^2 = 0.87$, respectively, $p < 0.05$) with extraction temperatures. With increasing extraction temperature, agar yield was positive correlated ($r^2 = 0.7$, $p < 0.05$) while gel strength was negatively correlated ($r^2 = -0.7$, $p < 0.05$) to sulphate content.

The agar yield with 1 and 1.5 h extraction time was significantly lower ($p < 0.001$) than the other extraction time. The agar yield with extraction times of 2, 2.5 and 3 h was not significantly different with

Table 4
Agar yield and properties (mean ± standard error) from *Gracilaria cliftonii* at extraction temperature of 70, 80, 90 and 100 °C.

	Extraction temperature (°C)			
	70	80	90	100
Agar yield (%db)	48.0 ± 0.6^{a}	51.9 ± 0.6^{b}	54.8 ± 0.2^{c}	60.6 ± 0.4^{d}
Gel strength (g/cm^2)	202.1 ± 17^{a}	246.7 ± 28^{a}	233.5 ± 18^{a}	186.8 ± 8^{a}
Melting point (°C)	85.9 ± 0.7^{a}	86.8 ± 0.1^{a}	87.0 ± 0.8^{a}	87.2 ± 0.7^{a}
Gelling temperature (°C)	37.6 ± 1.8^{a}	39.2 ± 1.3^{a}	38.8 ± 1.4^{a}	38.3 ± 0.8^{a}
Sulphate (%db)	5.7 ± 0.9^{a}	5.0 ± 0.8^{a}	5.8 ± 1.4^{a}	7.5 ± 0.6^{a}

Different letters (a, b, c) indicates significant differences at a level of $p < 0.05$.

Table 5
Agar yield and properties (mean ± standard error) from *Gracilaria cliftonii* at extraction time 1, 1.5, 2, 2.5 and 3 h.

	Extraction time (h)				
	1	1.5	2	2.5	3
Agar yield (%db)	38.2 ± 1.3^{a}	39.1 ± 1.5^{a}	54.8 ± 0.2^{b}	55.6 ± 0.2^{b}	56.7 ± 0.4^{b}
Gel strength (g/cm^2)	109 ± 2^{a}	146 ± 7^{b}	$138 \pm 18^{a,b}$	$126 \pm 10^{a,b}$	147 ± 17^{b}
Melting point (°C)	76.3 ± 5^{a}	85.9 ± 2^{b}	84.5 ± 1^{b}	84.4 ± 1^{b}	$82.0 \pm 1^{a,b}$
Gelling temperature (°C)	30.8 ± 1^{a}	34.0 ± 1^{b}	$33.1 \pm 1^{a,b}$	34.4 ± 1^{b}	$33.1 \pm 1^{a,b}$
Sulphate (%db)	7.8 ± 0.1^{a}	6.4 ± 0.6^{a}	7.9 ± 0.6^{a}	6.9 ± 1.4^{a}	6.7 ± 0.8^{a}

Different letters (a, b) indicates significant differences at a level of $p < 0.05$.

each other. Gel strength of agar with 1.5 and 3.0 h extraction time was significantly higher ($p < 0.05$) than with 1 h extraction time. Melting point of agar with 1 h extraction time was significantly lower ($p < 0.05$) than extraction time of 1.5, 2.0 and 2.5 h. Gelling temperature with 1 h extraction time was significantly lower than 1.5 and 2.5 h ($p < 0.05$) extraction time. Agar yield showed strong positive correlation ($r^2 = 0.8$, $p < 0.05$) with extraction time. With increasing extraction time, gelling temperature showed strong positive correlation ($r^2 = 0.9$, $p < 0.05$) with melting point.

3.2. Alkali treatment

3.2.1. Agar yield

At a given temperature, different NaOH concentrations had no influence ($p > 0.05$) on agar yield of *G. cliftonii* (Fig. 1). However, different temperatures at NaOH concentrations of 1–3% significantly affected the agar yield. At 70 °C, agar yield from *G. cliftonii* treated with 1% and 3% NaOH was significantly higher ($p < 0.05$) than at 60 °C. At 60 °C, agar yield from *G. cliftonii* treated with 2% NaOH was significantly lower ($p < 0.05$) than 70 and 80 °C. However, temperature had no influence ($p > 0.05$) on agar yields when *G. cliftonii* was treated with 5% NaOH.

3.2.2. Gel strength

The gel strength of agar from alkali treated *G. cliftonii* at different temperatures is shown in Fig. 2. Gel strength of agar at 60 °C, from 1% to 3% NaOH treated *G. cliftonii* samples was significantly higher ($p < 0.05$) than at 70 and 80 °C. In addition, gel strength of agar at 70 °C, from 2% NaOH treated samples was significantly higher ($p < 0.05$) at 80 °C. Gel strength of agar at 70 °C, from 5% NaOH treated *G. cliftonii* was significantly lower ($p < 0.05$) than at 60 and 80 °C. Only at 60 °C, alkali concentration up to 3% influenced ($p > 0.05$) the gel strength of agar. At 60 °C, gel strength of agar from *G. cliftonii* treated with 3% and 5% NaOH was significantly higher ($p < 0.05$) than 1% and 2% NaOH concentration. In addition, at 60 °C, gel strength of agar from samples treated with 2% NaOH was significantly higher ($p < 0.05$) than 1% NaOH. At 80 °C, gel strength of agar from 5% alkali treated *G. cliftonii* was significantly higher ($p < 0.05$) than other alkali concentrations. In addition, gel strength showed a strong positive correlation with alkali concentration ($r^2 = 0.96$, $p < 0.05$).

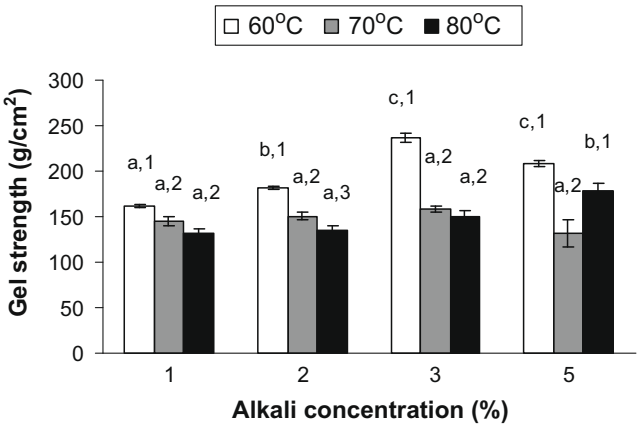

Fig. 2. Mean gel strength of agar (g/cm^2) from *Gracilaria cliftonii* treated with different alkali concentrations and temperatures of 60, 70 and 80 °C. Error bars represent standard error of the mean. Letters a, b, c represents difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between temperatures.

3.2.3. Sulphate content

Sulphate content of agar from alkali treated *G. cliftonii* at various alkali concentrations and temperature ranged between 2.16% and 3.01%db (Fig. 3). Alkali concentrations at different temperatures had no influence ($p > 0.05$) on the sulphate content of the agar.

3.2.4. Gelling temperature

The variations in gelling temperature of agar for alkali treated *G. cliftonii* samples at different temperatures are shown in Fig. 4. Gelling temperature of agar at 80 °C was significantly higher ($p < 0.05$) than at 60 and 70 °C from 1% NaOH treated *G. cliftonii*. In addition, gelling temperature of agar at 80 °C was significantly higher ($p < 0.05$) than at 70 °C from *G. cliftonii* treated with 5% NaOH. However, gelling temperature of agar at 70 °C was significantly higher ($p < 0.05$) than 60 and 80 °C from *G. cliftonii* treated with 2% and 3% NaOH. Except *G. cliftonii* treated with 3% NaOH,

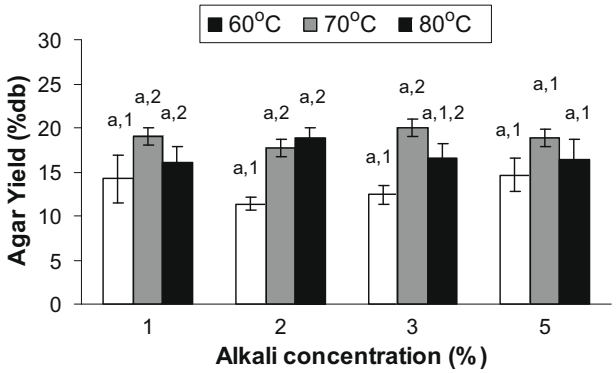

Fig. 1. Mean agar yield (%db) from *Gracilaria cliftonii* treated with different alkali concentrations and temperatures of 60, 70 and 80 °C. Error bars represent standard error of the mean. Letter a represents difference between alkali concentrations while numbers 1, 2 represents significant differences between temperatures.

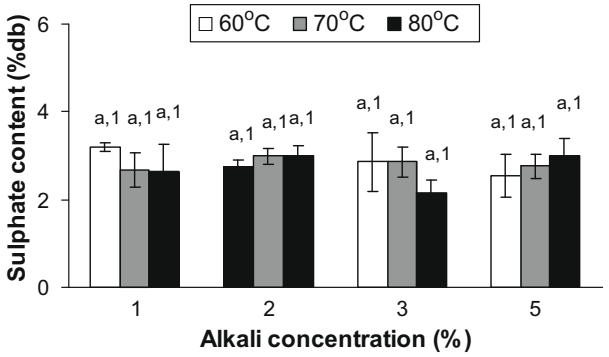

Fig. 3. Mean sulphate content (%db) of agar from *Gracilaria cliftonii* treated with different alkali concentrations and temperatures of 60, 70 and 80 °C. Error bars represent standard error of the mean. Letter a represents difference between alkali concentrations while number 1 represents significant differences between temperatures.

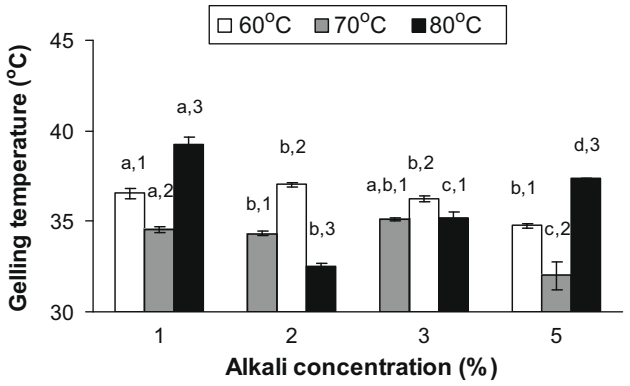

Fig. 4. Mean gelling temperature (°C) of agar from *G. cliftonii* treated with different alkali concentrations and temperatures of 60, 70 and 80 °C. Error bars represent standard error of the mean. Letters a, b, c represents difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between temperatures.

temperature significantly influenced ($p < 0.05$) the gelling temperature of agar. At 60 °C, gelling temperature of agar from 1% NaOH treated *G. cliftonii* was significantly higher ($p < 0.05$) than 2% and 5% NaOH. At 70 °C, gelling temperature of agar from 2% and 3% NaOH treated samples was significantly higher ($p < 0.01$) than 5% NaOH. At 80 °C, gelling temperature of agar was significantly different ($p < 0.05$) at all alkali concentrations.

3.2.5. Melting point

Melting point of agar was in the range 83.0–86.0 °C, with minimum observed for *G. cliftonii* treated with 3% NaOH at 60 °C and maximum for 1% NaOH at 80 °C (Fig. 5). Alkali treatment of *G. cliftonii* at different soaking temperatures did not influenced ($p > 0.05$) the melting point of agar.

4. Discussion

4.1. Agar extraction variables

It is critical to standardise the agar extraction process for each species in order to maximise its yield and simultaneously obtain a good quality agar (Freile-Pelegrin & Robledo, 1997b). The present study is the first attempt to test different variables in agar extraction process from *G. cliftonii* to optimise yield and to investigate their impact on agar properties.

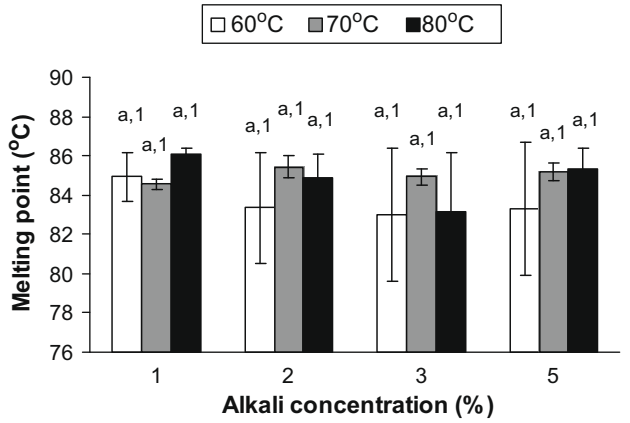

Fig. 5. Mean melting point (°C) of agar from *Gracilaria cliftonii* treated with different alkali concentrations and temperatures of 60, 70 and 80 °C. Error bars represent standard error of the mean. Letter a represents difference between alkali concentrations while number 1 represents significant differences between temperatures.

The purpose of soaking is to hydrate the seaweeds and ease the availability of soluble polysaccharides. Effects of soaking time and soaking temperature have not been reported for any *Gracilaria* species but different researchers over years have used different soaking times for other seaweeds prior to extraction (Arvizu-Higuera et al., 2008; Orduña-Rojas et al., 2008). The main constituents of seaweeds are polysaccharides, which are hydrophilic polymers. Water reacts with external and internal polysaccharides of seaweeds by hydrogen bonding. Besides water-holding capacity of seaweeds also varies according to the species (Jiménez-Escrig & Sánchez-Muniz, 2000). The longer soaking time (3 h) can result in diffusion of some agar into water resulting in lower yields. In addition, negative correlation between agar yield and sulphate content with increase in soaking time indicates alteration in agar structure as sulphate forms important part of agar molecular chain. The role of sulphate on agar yield due to change in agar structure has been reported in various studies (Armisen & Galatas, 2000; Falshaw et al., 1999; Marinho-Soriano et al., 1999; Orduña-Rojas et al., 2008). Longer soaking time also influenced gelling temperature of agar, which was highest at 3 h of soaking in our studies. The high gelling temperature is also related to the change in agar structure due to longer soaking time. The gelling temperature is reported to be positively correlated to the methoxyl content of agar (Guiseley, 1970; Rebello, Ohno, Ukeda, Kusunose, & Sawamura, 1997) which in present study was not determined.

The soaking temperature can also influence the agar yield and properties as the average ambient temperature during the culture of *Gracilaria* varies from various geographic region and time of the year. Different soaking time strongly influenced the agar yield, gelling temperature and sulphate content of agar. At higher soaking temperatures (35 and 40 °C) lower yield could be due to some diffusion of agar in water. The high yield at 30 °C is related to changes in agar structure thus allowing the release of most polysaccharides when extracted at this temperature. The polysaccharides have intracellular hydrogen bonding which can be broken due to mild heat thus altering the structure. This can also explain the high gelling temperature of agar with soaking temperature of 30 °C as compared to 40 °C. The gelling temperature of agar is reported to increase with increase in methoxyl content (Andriamananatonio et al., 2007; Duckworth & Yaphe, 1971; Guiseley, 1970). Higher temperature (40 °C) can rupture the agar-methoxyl bonding resulting in lower gelling temperature. In addition, higher sulphate content with higher soaking temperature can affect the gelling temperature as reported in various studies (Andriamananatonio et al., 2007; Arvizu-Higuera et al., 2008; Craigie & Jurgens, 1989; Navarro, Flores, & Stortz, 2007; Orduña-Rojas et al., 2008). The increase in sulphate content with soaking temperature can be related to heat induced changes in the molecular structure of agar.

Effects of seaweed–water ratio during agar extraction process on agar properties has not been published for any *Gracilaria* species but different researchers over years have used different ratios for agar extraction (Araño, Trono, Montaño, Hurtado, & Villanueva, 2000; Arvizu-Higuera et al., 2008; Freile-Pelegrin & Murano, 2005; Orduña-Rojas et al., 2008; Tako et al., 1999). Agar yield and gel strength were significantly different with different volumes of water for same amount of *G. cliftonii*. The greater the volume of water better is the swelling of seaweed thus, allowing the agar to be extracted easily. Dried marine algae can swell to about 20 times of their dry matter volume when exposed to water (Jiménez-Escrig & Sánchez-Muniz, 2000). Agar yield with seaweed–water ratio of 1:200 was lower and resulted in higher gel strength, which may be due to the excess of water resulting in diffusion of agar. The higher gel strength with seaweed–water ratio of 1:200 could be attributed to the changes in agar structure and can be explained with low sulphate content (Andriamananatonio et al., 2007; Arvizu-Higuera et al., 2008; Craigie & Jurgens, 1989; Duck-

worth & Yaphe, 1971; Nunn, Parolis, & Russell, 1973; Orduña-Rojas et al., 2008). Gelling temperature showed a negative correlation with sulphate and is reported to be affected by the methoxyl content in agar (Andriamananatonio et al., 2007; Arvizu-Higuera et al., 2008; Craigie & Jurgens, 1989; Navarro et al., 2007; Orduña-Rojas et al., 2008). The agar structure change due to increasing water volume can result due to change in location of methoxyl group and sulphate in agar structure (Craigie & Jurgens, 1989; Lloyd, Dodgson, Price, & Rose, 1961). In addition, melting points negative correlation to gel strength also indicates change in structure and may be due change in pyruvic acid content of agar. Melting point is reported to be positively correlated to the pyruvic acid content and alters the structure of agar (Young, Duckworth, & Yaphe, 1971).

Different extraction temperatures significantly influenced the agar yield of *G. cliftonii* but also indicated a significant amount of agar is extracted even at low temperature (70 °C). The agar extraction temperature from *Gracilaria* species is reported to be 85–100 °C (Arvizu-Higuera et al., 2008; Marinho-Soriano & Bourret, 2005; Meena, Prasad, & Siddhanta, 2006; Orduña-Rojas et al., 2008; Santelices & Doty, 1989) but there is no literature reporting influence of extraction temperature on agar yield and properties. Various authors have reported alkali treatment temperatures of 80–100 °C for *Gracilaria* prior to extraction and observed significant decrease in agar yield due to the process (Armisen & Galatas, 2000; Falshaw et al., 1999; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Marinho-Soriano, 2001). In the present study, agar yield observed at different extraction temperatures can be correlated to the decrease in agar yield during alkali treatment of *Gracilaria*. Agar yield of 48%db at extraction temperature of 70 °C indicate that considerable amount of agar is being extracted at low temperatures therefore while treating *Gracilaria* with alkali at even 70 °C, agar gets extracted and can be lost when discarding excess alkali after the treatment. The agar yield was different for various extraction temperatures with maximum at 100 °C which is in agreement with previous studies (Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b). Extraction temperature's strong positive correlation with agar yields indicates its strong influence on agar extraction. In addition, melting point also showed positive correlation with extraction temperature indicating the alteration of structure due to higher temperatures in extraction process though the increase is not significant. Gel strength's negative correlation with sulphate and positive correlation with gelling temperature confirms the change in structure due to conversion of L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Duckworth et al., 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas et al., 2008).

In present study, extraction time was found to be as one of the most important variables in the extraction process as agar yield and its properties (except sulphate content) showed significant differences at different extraction times. Agar yield was significantly lower with extraction time of 1 and 1.5 h, which is due to incomplete agar extraction. All the properties were affected with 1 h extraction time indicating that minimum time is required to complete the agar extraction to have desirable properties. The extraction time for maximum yield was 2–3 h, which is in confirmation with various agar-based studies. For example, Thomas and Krishnamurthy (1976) studied the effect of extraction time (1–6 h) on *G. edulis* and found maximum yield with 2–4 h extraction time, Arvizu-Higuera et al. (2008), also reports the maximum agar yield with 2.5 h extraction time in *G. vermiculophylla*.

4.2. Alkali treatment

Not all the combinations of alkali concentrations and temperatures had a significant effect on agar characteristics but physical and chemical characteristics of the agar were improved as compared to native agar. However, the agar characteristics obtained were similar to those reported for native agar from *G. cornea* (Freile-Pelegrin & Robledo, 1997a). The agar yields obtained after alkali treatment of *G. cliftonii* were higher than those required by the industry (>8%db) (Armisen, 1995), but were lower than the reported agar yield of 52%db (Byrne et al., 2002). The decrease in agar yield of *G. cliftonii* when treated with alkali as compared to native agar can be due to agar diffusion into the water and/or due to the difference in extraction temperatures of agar used in present study. In addition, decrease in agar yield due to alkali treatment compared to native agar suggests that different alkali concentrations in combination with soaking temperatures could have resulted in some degradation of the polysaccharide (Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b).

The agar yield of *G. cliftonii* treated with high alkali concentrations at different temperatures was similar to that observed for *G. edulis* (21.8%db) (Durairatnam, 1987) and *G. verrucosa* (21.1%db) (Hurtado-Ponce & Umezaki, 1988). At same NaOH concentrations the agar yields were similar to *G. cornea* at 80 °C (Freile-Pelegrin & Robledo, 1997b). Agar yield obtained at 80 °C was also similar to *G. cervicornis*, *G. blodgetti* and *G. crassissima* (13–26%db) at 3% and 5% NaOH concentrations. The difference in agar yield could be related to the differences in used variables like alkali concentration, temperature and time for extraction process (Armisen & Galatas, 1987; Arvizu-Higuera et al., 2008).

The gel strength achieved with different alkali concentrations was similar to the gel strength for native agar from various other *Gracilaria* species (Falshaw et al., 1999; Freile-Pelegrin & Murano, 2005; Orduña-Rojas et al., 2008; Santos & Doty, 1983). Freile-Pelegrin and Robledo (1997b) reported increase in gel strength in *G. cornea* after treatment with 3% and 5% NaOH concentrations than gel strength of native agar. In the present study, there was no significant improvement in the gel strength with alkali treatment at a given temperature, which can be due to difference in extraction process and/or species. However, the strong positive correlation between the gel strength and alkali concentration is in agreement with the various studies (Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b) and can be due to the elimination of the sulphate ester at C-6 of the L-galactose to increase the 3,6-anhydro-L-galactose content thereby improving the gelling properties (Murano, 1995).

Alkali treatment converts L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Duckworth et al., 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas et al., 2008), which is responsible for the enhancement of the gel forming ability but in *G. cliftonii* this conversion may not occur. Sulphate content was lower than other *Gracilaria* species but no relation was observed with gel strength with different alkali concentrations at any temperature.

The variation in gelling temperature due to the treatment with the various alkali concentrations and at different soaking temperatures can be related to molecular weight and molecular weight distribution in agar as these parameters strongly affect the gelling properties (Freile-Pelegrin & Murano, 2005). In addition, the methoxyl content and location is reported to affect the gelling temperature of agar (Andriamananatonio et al., 2007; Guiseley, 1970; Rebello et al., 1997) which was not analysed in present study. However, gelling temperature of agar was similar to *G. cervicornis* (Freile-Pelegrin & Murano, 2005), *G. corticata* (Oyieke, 1993), *G. crassa* (Oyieke, 1993), *G. millardetii* (Oyieke, 1993) and *G. salicornia* (Oyieke, 1993).

The melting point was not affected by the alkali concentration at different temperatures but was within the range specified by US Pharmacopoeia (80–85 °C) suggesting possible applications for food and pharmaceutical industry, especially for products that require sterilization. In addition, melting point was similar to *G.*

tenuistipitata (Montaño, Villanueva, & Romero, 1999), *G. arcuata* (Montaño et al., 1999), *G. corticate* (Oyieke, 1993), *G. crassa* (Oyieke, 1993), *G. millardetii* (Oyieke, 1993) and *G. salicornia* (Oyieke, 1993). Melting point is reported to be positively correlated to pyruvic acid content of agar (Young et al., 1971), however further investigation is required as this factor was not analysed in present study.

The present result confirms that agar yield is influenced by all the variables used and tested during agar extraction process. It also shows that all the agar properties can be manipulated by manipulating soaking time and temperature, weed–water ratio, extraction time and temperature in the extraction process. At a given temperature, there was no effect of alkali treatment on agar properties suggesting different requirements of alkali concentration and temperature for *G. cliftonii*. The results obtained suggest *G. cliftonii* treated with different alkali concentrations at different soaking temperatures considerably reduce the agar yield. However, since the agar characteristics can depend on other extraction variables and internal characteristics of the species it is impossible to establish a general extraction method for agarophytes to obtain a high quality agar for industrial use (Freile-Pelegrin & Robledo, 1997b). In our study, the gel strength and sulphate content obtained from agar treated with 3% NaOH at 70 °C are in the range required by the Asian food industry (gel strength for striped form agar in range 150–450 g/cm^2 (1.5% w/v gel) and sulphate content less than 4%db, usually 1.5–2.5%db (Armisen, 1995). In addition, similar gel strength can be obtained by either manipulating the extraction process variables or alkali treatment. It is recommended that dry *G. cliftonii* is soaked for 1 h at 30 °C with seaweed–water ratio of 1:150 and extraction of agar in boiling water at 100 °C for 3 h to obtain maximum yield and higher quality.

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